STUDIES ON SPOROTHRIX SCHENCKII HEKTOEN AND PERKINS AS A SAPROPHYTE IN NATURE

R. L. Sautter and P. A. Volz

Mycology Laboratory, Biology

Eastern Michigan University, Ypsilanti, Michigan 48197

Introduction: The filamentous fungus Sporothrix schenckii Hektoen and Perkins has the ability to grow on decaying vegetation in nature while as a parasite the organism can cause mycoses in animals and in man (Baker, 1971; Jungerman and Schwartzman, 1972). Of 250 native and newly naturalized flowering plant species found in Taiwan, 114 supported no growth, 65 supported moderate growth, 37 supported good growth while 35 species induced abundant mycelial and conidial production of S. schenckii as a saprophyte (Volz and Pan, 1976).

Reports are limited on the ability of the organism to infect living plant tissue. Benham and Kesten (1932) suggested the capability of S. schenckii to infect carnations causing bud wilt. Although S. schenckii has been isolated many times from plants in relation to human epidemics of sporotrichosis (Emmons et al., 1977; Foerster, 1926; Rippon, 1974), the species was never substantiated as a causative agent in plant disease. The purpose of this study is to examine the phytoalexins stimulated by Sporothrix schenckii to further identify the relationship of the organism to higher plants.

Literature Review: The stimulation of phytoalexins by pathogenic and nonpathogenic organisms is indicative of infectivity in plants (Varns, 1971). Phytoalexins are phenolic compounds that exhibit antifungal activity in living plants. Müller (1958) was the first to postulate the existence of fungalstatic compounds while studying potato tubers with virulent and avirulent races of Phytophtora infestans in late blight of potato. Since then additional compounds have been isolated from plants in response to fungal infection. Plant systems examined included sweet potato (Kim and Urtani, 1974; Urtani, 1971, 1963), orchid (Fisch et al., 1973, 1972), carrot (Condon and Kuc, 1960; Hampton, 1962), potato (Sato et al., 1968; Tomiyama et al., 1968a; Varns, 1970; Varns, Kuc and Williams, 1971), barley, green pepper, rice, turnip (Metlitskii and Ozeretskovskaya, 1968), bean (Pierre, 1971; Pierre and Bateman 1967), soybean (Keen et al., 1971), alfalfa (Higgins and Millar, 1968), French bean (Deveral et al., 1968; Pierre and Bateman, 1967; Rahe et al., 1969), safflower (Allen and Thomas, 1971a, b, c), strawberry (Mussell and Stoeple, 1971), chili pepper (Bhullor et al., 1972), pepper fruits (Müller, 1958; Stoessel et al., 1972; Van den Ende, 1969), parsnip (Johnson and Brannan, 1973). Fungalstatic compounds isolated from plants include ipomearone (Metlitskii and Ozeretskovskaya, 1968), Pisatin (Cruickshank and Perrin, 1963, 1961,

1960), isocoumarin (Condon and Kuc, 1960), phaseolin (Cruickshank and Perrin, 1963), phytuberin and rishitin (Varns, 1971; Tomiyama et al., 1968b), xanthotoxin (Johnson and Brannan, 1973), and 6a-hydroxyphaseollin (Keen et al., 1971).

Phytoalexins, like antibodies, seem to be stimulated in response to infection as well as to the introduction of foreign substances. Pathogens as well as nonpathogens and chemical substances are capable of stimulating a response in plant tissue. Inhibiting substances can be stimulated by a wide variety of organisms and chemicals, the substances are nonspecific in their action. In addition, the same compound may be produced in response to several different organisms. The reactions can vary according to the amount of compound produced or in the toxicity level against the invader (Varns, 1971). The rate of phytoalexin production by the host is also important in controlling the invader. Phytoalexin response of both resistant and succeptable host varieties are apparently similar but the former produces phytoalexins more quickly and possibly in larger quantities (Müller and Borger, 1940; Varns, 1971).

Classical theories on the action of phytoalexins indicate that a stimulation in the plant by the primary fungal invader occurs, and that the response toward this stimulation results in the death of the invader. Pathogens have the ability to degrade phytoalexins and are unaffected by their action. Nonpathogens are unable to degrade phytoalexins, and growth is prevented (Higgins and Millar, 1970; Wit-Eshove, 1969). Infection of potato cultivars by strains of Phytophtora infestans produce antifungal compounds rishitin and phytuberin (Sato et al., 1968; Varns et al., 1971). Hypersensitive resistance is controlled by a series of major genes called "R genes" that permit some races of Phytophthora infestans to become infective and others not infective (Friend, 1973). The amount of phytoalexin accumulated in the plant depends on the type of R-gene present and the parasite race introduced in the host (Sato et al., 1968; Varns et al., 1971).

The role of phytoalexins in disease resistance in plants has also been examined. Kiraly et al. (1972) concluded that on death or inhibition of the fungus, endotoxins are released which stimulate phytoalexin production. This would place the phytoalexin role in plant resistance at the point of inhibiting secondary infection (Van der Plank, 1975). Saprophytic organisms existing on the surface of a plant would remain suppressed. Phytoalexins as plant defense mechanisms have attracted increased attention by researchers (Christensen, 1969; Higgins and Millar, 1970; Sato et al., 1968; Varns et al., 1971; Wit-Eshove, 1969). It is apparent that phytoalexins act as inhibitors of incompatable potential invaders and show ineffectiveness against compatable or virulent pathogens.

Materials and Methods: Carnation buds, potential hosts for Sporothrix schenckii (Benham and Kestan, 1932), were injected with

viable conidia collected from 4 week old colonies grown on Sabouraud dextrose agar. Control buds received ½ml 1% sterile sucrose solution and additional control buds received no treatment. All carnation buds were examined for the presence of phytoalexins. Inoculated excised buds were incubated at room temperature in sterile petri plates containing sterile filter paper. All buds were carefully examined for the presence of other fungal species other than S. schenckii, and buds found with contaminants were eliminated. Buds with S. schenckii induced deterioration were collected and placed in a Waring blender in 95% ETOH. After blending, particles were extracted in a sohxlet extractor in 95% ETOH. Control groups were extracted in the same manner. Extracts were purified by paper chromatography using n-butanol, acetic acid, and water, 4:2:1 v/v/v (Hampton, 1962). Chromatographic spots were detected with ultraviolet light. Thin layer chromatography further purified the extracts.

The antifungal activity of S. schenckii in each chromatographic spot was examined. The spots were removed and placed on dialysis tubing coated with Sabouraud dextrose agar containing streptomycin. At the edge of the spot an inoculation was made of S. schenckii conidia. Hyphae were allowed to grow at room temperature to partially cover the chromatographic spot. Undisturbed hyphal apices with the supporting dialysis tubing were removed and mounted on slides with lactophenol cotton blue (Volz and Niederpruem, 1968).

Studies on the ability of potato tubers to respond toward S. schenckii were also initiated. Twenty grams of peeled, sliced potato tubers were aseptically placed in sterile petri plates containing filter paper. Inoculations of 2 ml conidial suspension were added to each potato test. Control potato plates were treated with 2 ml sterile sucrose solution. At 4 days incubation the test potatoes contained a heavy growth of S. schenckii. Extraction of phytoalexins followed using 20 g samples from each plate. Previously described assay methods were used to measure antifungal activity of the extracts. Extract purification by thin layer chromatography was carried out (Lyon, 1972; Varns et al., 1971). Ethanol extracts were further treated with water and chloroform to final volume 3:2:1 v/v/v of ethanol, water and chloroform. The mixture was then shaken in a separatory funnel 1 minute and allowed to separate into phases for 1 hour. The chloroform phase was removed. The aqueous phase twice extracted with the same volume of chloroform, and all chloroform extractions were combined. Chloroform phases of both control and test systems were completely evaporated and redissolved in acetone. The extract was applied to thin layer silica gel chromatographic plates (Kodak) using a solvent system of cyclohexane / ethyl acetate, 1:1 v/v. Detection of spots occurred by spraying with chloroform saturated with antimony chloride. Similar plates using chloroform / ethyl acetate 1:1 v/v were used. Each plate was examined under UV light before and after spraying with the developer. Photographs of the plates were taken.

Results: Carnation buds were selected for study due to their capability of infection by S. schenckii (Benham and Kesten, 1932). Potato tubers were selected to test the ability of S. schenckii to attack a common food supply grown in soil vulnerable to soil microbes. Paper chromatography of extracts revealed a variation of migrations in both carnation buds and potato tubers infected with S. schenckii as compared to their respective controls. Carnation chromatographic spots I and II were found in carnation control group (CC), carnation fungal contaminant control (CFC), and the carnation test (CT) while spots III, IV and V were found in both CFC and CT. Spot (a) was found in the test (CT) and control (CC) groups, and spot (b) was located only in the control (CC). All spots from each extract fluoresced under 254 nm UV light. Only spot V found in CT and CFC chromatograms fluoresced red instead of yellow (Tables I,V).

Extracts from infected potato tubers contained 4 migratory spots while extracts from uninfected potatoes exhibited 3 separations with paper chromatography (Table II). Spots designated as 1,2,3, and 4 corresponded to an increase in Rf value. Migration 4 was present only in the infected potato tubers while separations 1,2, and 3 were found in both inoculated and control tissue in apparently equal amounts.

Observations on hyphal growth of S. schenckii in association with most chromatographic spots demonstrated some inhibitory response to the growth rate compared with control. Abnormal growth patterns of S. schenckii hyphae grown on dialysis membrane in contact with inhibitory chromatographic migrations included no growth or independent growth of an isolated individual filament. Undisturbed colony sections grown on dialysis tubing covered agar, mounted in lactophenol cotton blue, indicated normal hyphal growth including branching patterns of parallel hyphae, and parallel hyphae tightly organized in a pyramid arrangement terminating in a leader hypha.

Thin layer silica gel chromatography was selected to clarify variation noted in extracts of control and inoculated tissue. With chloroform and ethyl acetate as the migratory phase in the carnation study, isolated extracts corresponded to those found in the previous carnation study (Table III). Migration I appeared only in the CFC group while II and IV appeared in CFC and CT groups. Spot III was present in CC, CFC, and CT groups.

Thin layer chromatography with carbon tetrachloride and ethyl acetate as the migratory phase was made with the potato tuber study (Table IV). The potato chromatographic spots migrating at the same Rf value when viewed under UV light at 254 nm before chemical spraying indicated that the predominant spot occurs in the uninoculated potato (PuI). After spraying the chromatograph with chloroform saturated with antimony trichloride (Lyon, 1972; Varns et al., 1971), the predominant spot was noticed in inoculated potato tubers (PiI). A change in color in the control extract upon treatment with the developer was also noted.

Carnation extracts produced numerous migratory separations in thin layer chromatography (Table V). The carnation control group (CC) did not produce many migrations. Extracts CT and CFC were composed of similar migrations, however, variation occurred in treating the plates with UV radiation and with the developer of chloroform saturated with antimony trichloride. Each spot found in the CT group was found in the CFC group with the exception of migration II.

Discussion: Sporotrichosis is a disease of importance to man causing cutaneous, subcutaneous, and systemic involvements. The causal organism, Sporothrix schenckii, is found in nature associated with soil containing high levels of organic matter and with living plants. Benham and Kesten (1932) carried out early studies with the fungal isolate and demonstrated infectivity in carnation, rose, barberry, and June grass. In the current study, host plant phytoalexin production and fungal infectivity were examined.

A variation in separatory migrations was noted in extracts obtained from S. schenckii infected carnation buds. The control demonstrated a similar pattern to the carnation test except spot (a) was found in CT and not in CFC (Table I). Plants inoculated with sterile sucrose or sterile sucrose containing conidia had more migrations compared to the carnation control. The plant capable of responding to either physical injury or S. schenckii infection is clearly evident. Both the CFC group and CT group contained phytoalexins presumably in response to a surface contaminant or S. schenckii infection.

Carnation spot (b) was only found in extracts from the carnation control (CC) which possibly could be a precursor substance which was changed slightly on growth of surface contaminants or introduction of S. schenckii. Migratory separations unique to tissue infected with S. schenckii were not found. The quantity or size of spots detected indicate that the same amount of phenol was present from both CT and CFC groups and a variation in size and number of spots was only slightly significant when compared to the CC control. Apparently S. schenckii is incapable of stimulating an additional response in carnation buds.

Hyphal growth rates and hyphal morphology were observed with S. schenckii grown in contact with chromatographic separations obtained from carnation. Phenolic antifungal activity of the spots was detected by growth inhibition and abnormal hyphal morphology. An inhibitory response was noted more frequently with CT and CFC extracts than from CC. Corresponding spots from each group demonstrated varying degrees of activity. Migration I, present in all three extracts, presented similar colony inhibition while microscopically greater variation occurred with greatest inhibition in CT. A similar situation existed with CT III and CFC III, with CFC III having the greatest inhibition. Migration groups CT and CFC displayed greater inhibitory responses than did group CC. The

response in carnation is attributed to the presence of S. schenckii in the host plant or possibly an undetected surface contaminant.

Thin layer chromatography of carnation extracts was employed to note possible additional separations not found in the other selected method. The chloroform and ethyl acetate solvent system revealed four spots upon UV irradiation (Table III). Migration I appeared only in the CFC group while II and IV were present both in CFC and CT groups. Separation III appeared in all groups, the controls and the test groups. Carbon tetrachloride and ethyl acetate solvent system revealed 5 separations in the test material (Table V). The control group (CC) displayed a faint migration which was present in the other extracts. The carnation contaminant control (CFC) produced spots I, III, IV, and V. Only spot II was unique to the test group which was detected at Rf 0.44 after developing the chromatograph with chloroform saturated with antimony trichloride. Paper chromatography omitted some separatory spots and one missed was unique to carnation buds infected with S. schenckii.

Potato tubers inoculated with S. schenckii clearly indicated with paper chromatography that potatoes were stimulated to produce phenolic compounds. Chloroform and ethyl acetate as the moving phase in thin layer chromatography produced three migrations. Spots I and III were found in both control and test material while other spots were present only in the inoculated tubers indicating the production of phenolic compounds. The carbon tetrachloride ethyl acetate solvent system isolated only one migration each on UV irradiation in control (PuI) and test potato (PiI) tuber material inoculated with S. schenckii. A faint blue spot was noticed at Rf 0.56 of the infected tubers while a strong blue spot was noticed in the uninfected tubers. After spraying with the developer, chloroform saturated with antimony trichloride, the migration from infected potato tubers was large in size and blue in color. The separation from uninoculated tissue was beige and moderate in size.

Potatoes have been known to produce two phytoalexins, rishitin and phytuberin (Lyon, 1972; Sato et al., 1968; Varns et al., 1971). With the same solvent system and thin layer chromatography, Varns et al. (1971) and Lyon (1972) found rishitin and phytuberin to migrate at Rf values of 0.3 and 0.7 respectively. The compound detected in potato tubers infected with S. schenckii appears to be rishitin primarily due to coloration and migratory properties.

Phytoalexins are universally found in the plant kingdom. Both carnation and potato responded to infection with S. schenckii by producing phytoalexins. Benham and Kesten (1932) reported S. schenckii capable of producing carnation bud wilt. According to Varns et al. (1971), Christensen (1969), and Higgins and Millar (1968), susceptability is a result of a suppression of the plant's response. Organisms normally pathogenic for a specific plant, carrying the correct R gene, are capable of suppressing the formation of phytoalexins in order to attack the host. Nonpathogens are

not capable of suppressing the plants response and are thus inhibited. Sporothrix schenckii is not a pathogen of potatoes since the fungus is inhibited by the phytoalexins and apparently it can not break down the complex chemical structures. A mild variation in inhibition was noted between CFC and CT in the carnation studies which would indicate the possibility of S. schenckii is a pathogen of carnations.

Table I

Compounds Isolated from Phytoalexin Studies of Carnation Buds Inoculated with Sporothrix schenckii

Spot designation	Rf value	Reaction with UV illumination at 254 nm
I	0.17	yellow
II	0.30	yellow
III	0.42	yellow
IV	0.49	yellow
V	0.57	red
a	0.043	yellow
b	0.54	yellow

Table II

Compounds Isolated from Phytoalexin Studies of Potato Tubers Inoculated with Sporothrix schenckii

Spot designation	Rf value	Reaction with UV illumination at 254 nm
I	0.19	yellow
II	0.23	yellow
III	0.34	yellow
IV	0.91	blue

Table III

Separations of Carnation Extracts Using
Chloroform and Ethyl Acetate as the Migratory Phase

Spot designation	Rf value	Reaction with UV illumination at 254 nm
I	0.24	red
II	0.50	red
III	0.62	red
IV	0.72	red

Table IV

Potato Extracts Purified by Thin Layer Chromatography
Using Carbon Tetrachloride and Ethyl Acetate
as the Migratory Phase

Spot designation	Rf value	UV illumination and spot size	Antimony trichloride in chloroform, and spot size
PiI	0.56	light blue, small	strong blue, large
PuI	0.56	strong blue, large	beige, small

Table V

Carnation Extract Separations in Thin Layer
Chromatography with Carbon Tetrachloride / Ethyl Acetate
as the Migratory Phase

Spot designation	Test Group appearance	Rf value	UV illumination at 254 nm	Developer reaction
I	CFC, CT (faint)	0.26	red	beige
II	CT	0.44	absent	beige
III	CFC, CT	0.59	red	absent
IV	CFC, CT	0.65	absent	beige
V	CFC, CT, CC (faint)	0.71	absent	beige

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